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Acetylcholine-mediated neurotransmission within the nucleus raphe magnus exerts a key role in the organization of both interictal and postictal antinociception

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ABSTRACT

The role of the acetylcholine-mediated system in the organization of postictal antinociception was investigated. For this purpose, nicotinic and muscarinic cholinergic receptor antagonists were microinjected into the nucleus raphe magnus (NRM), a key structure of the endogenous pain inhibitory system. After the tail-flick test baseline recording, male Wistar rats ($N = 8$ per group) were submitted to stereotaxic surgery for the introduction of a guide cannula aiming at the NRM. Five days after surgery, atropine or mecamylamine (1 μg/0.2 μL, 3 μg/0.2 μL, or 5 μg/0.2 μL) was microinjected into the NRM. The tail-flick withdrawal latency was recorded immediately after peripheral treatment with pentylenetetrazole (PTZ) (64 mg/kg), in two different interictal time windows, and for 130 minutes after the last seizure evoked by intraperitoneal injection of PTZ. The blockade of GABA-mediated Cl^- influx caused tonic–clonic convulsions in all animals followed by sustained postictal antinociception lasting 110 minutes after seizures; the nociceptive threshold was also found to be high in interictal periods. Pretreatment of the NRM with either atropine or mecamylamine antagonized both interictal and postictal antinociception, suggesting the involvement of cholinergic mechanisms recruiting muscarinic and nicotinic cholinergic receptors of the NRM in the organization of tonic–clonic seizure-induced antinociception.

1. Introduction

Several investigations have been performed to elucidate the neuroanatomical and neurochemical bases of analgesia and the neural substrates involved in epileptic activity [1–9].

The postictal state is an immediate consequence of seizures [10], in which it is possible to detect oscillations of nociceptive thresholds [11–15]. It has been observed that during the postictal state, patients experience delusions, agitation, aggressive behavior [7], and cognitive symptoms such as attentional deficits and concentration impairments [8]. Paresis may also occur, signaling the location of seizures in the contralateral motor cortex [10].

Previous studies have demonstrated antinociceptive processes in experimental models of epilepsy in both human- [16] and animal [11,12]-based models. Among several approaches used in experimental neurology to induce seizures, the pharmacological model of epilepsy using pentylenetetrazole (PTZ), a GABAergic noncompetitive antagonist that does not interact directly with GABA receptors, but blocks GABA-mediated Cl^- influx, is used widely [2,17]. The intraperitoneal injection of PTZ in rats causes tonic–clonic seizures consistently followed by increases in the nociceptive threshold [5,18–22].

Mesencephalic structures, such as the periaqueductal gray matter (PAG) and nuclei of the ventromedial medulla oblongata, in particular the nucleus raphe magnus (NRM), have been suggested to play important roles in the control of supraspinal pain [1,23]. However, other nuclei of the endogenous pain inhibitory system, such as the dorsal raphe nucleus, rich in serotonin, and also the locus coeruleus, an important noradrenergic nucleus of the brainstem, have been implicated in tonic–clonic seizure-induced antinociception [24,25].

The first evidence suggesting involvement of the NRM in postictal analgesia was demonstrated by neurochemical damage of the NRM resulting in a significant decrease in postictal antinociception [3]. Raphe–spinal pathways originating in the NRM reach the gelatinous substance of the spinal cord, inhibiting noxious inputs. Previous evidence has demonstrated that muscarinic and nicotinic receptors are involved in the control of perception of nociceptive stimuli in both animal trials and human models of pain [6,26].

Published reports have described the involvement of endogenous opioid receptors [11,12] in hypoalgesic processes following seizures. The organization of postictal antinociception may involve a μ_1-opioid receptor-mediated mechanism in the central nucleus of the inferior colliculus [24], an endocannabinoid-mediated system of the

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ventrolateral columns of the PAG [14], and a serotonin-mediated system involving both the dorsal raphe nucleus [5] and the reticularis gigantocellularis/paragigantocellularis nuclei [3]. There is also evidence that acetylcholine may be involved as a neurotransmitter in postictal analgesia, suggesting a critical role for muscarinic and nicotinic cholinergic receptors in the organization of this interesting antinociceptive process [20].

In fact, both cholinergic and noradrenergic inputs to the NRM have been described to exert a remarkable role in the modulation of pain [9,27]. In addition, another report provided evidence that there is an excitatory connection between the PAG and the NRM. The results indicate that activation of this pathway can induce analgesia [28]. Acetylcholine is an excitatory neural transmitter at the NRM [27], and findings suggest there is another brainstem structure that sends inputs to the NRM [29]: the pedunculopontine tegmental nucleus, rich in choline acetyltransferase-containing neurons [19,28–32]. Therefore, the present study investigated the role of cholinergic mechanisms of the NRM in the elaboration of interictal and postictal antinociceptive processes that follow seizures caused by the systemic GABAergic dysfunction induced by PTZ.

2. Methods

2.1. Subjects

Male Wistar albino rats ($N = 8$ per group), weighing between 200 and 250 g, from the animal care facility of the Campus Universitarius of the University of São Paulo (USP) in Ribeirão Preto were used. These animals were housed in groups of four in a plexiglass-walled cage, with free access to food and water throughout the experiment. The room temperature was controlled (22 ± 1 °C), and a light/dark cycle (07:00–19:00 hours lights on) was maintained. All protocols were used in compliance with the recommendations of the Brazilian Society for Neuroscience and Behaviour (SBNeC), as well as with the recommendations of the Committee for Ethics in Animal Experimentation (CEUA) of the Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP) (Proc. 174/2005), which are in accordance with the Animal Research Ethics guidelines adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2. Stereotaxic surgery

The animals were anesthetized with sodium pentobarbital (45 mg/kg, ip) and fixed in a stereotaxic frame (David Kopf, USA). A stainless-steel guide cannula (o.d. 0.6 mm, i.d. 0.4 mm) was implanted in the brainstem, aimed at the NRM. The upper incisor bar was set 3.3 mm below the interaural line such that the skull was horizontal between the bregma and lambda landmarks. The guide cannula was vertically introduced using the following coordinates, with the bregma serving as the reference for each plane: anteroposterior, −10.5 mm; mediolateral, 0.0 mm; dorsoventral, 9.2 mm. The guide cannula was fixed to the skull by means of acrylic resin and two stainless-steel screws. At the end of the surgery, each guide cannula was sealed with a stainless-steel wire to protect it from obstruction. After the surgery, each animal was treated with an intramuscular injection of 60.000 IU of penicillin G benzathine and a nonsteroidal anti-inflammatory drug (banamine meglumine at 2.5 mg/kg).

2.3. Nociceptive tests

Each animal was individually placed in a restraining apparatus, and its tail was inserted into a heating sensor (tail-flick Analgesia Instrument; Stoelting, IL, USA). The heating sensor functions such that calorimetric progressive elevation is automatically interrupted at the moment when the animal removes its tail from the apparatus. The current raises the temperature of the coil (Ni/Cr alloy, 26.04 cm in length × 0.02 cm in diameter) from room temperature (approximately 20 °C) at the rate of 9 °C/second [33]. A small current intensity adjustment could be performed at the beginning of the experiment to obtain three consecutive tail-flick latencies (TFLs) between 2.5 and 3.5 seconds. If the animal did not remove its tail from the heater within 6 seconds, the tail-flick device was turned off to prevent damage to the skin. Baseline tail-flick test measurements were obtained before stereotaxic surgery.

Three baseline measurements of control TFLs were taken at 5-minute intervals. Animals (independent groups) received central microinjections of saline, atropine, or mecamylamine at different concentrations (1 μg/0.2 μL, 3 μg/0.2 μL and 5 μg/0.2 μL) in the NRM ($n = 8$); after 5 minutes animals received an intraperitoneal injection of PTZ (64 mg/kg). Nociceptive thresholds were recorded before and after peripheral or central injections of drugs, immediately after the last incidence of tonic–clonic seizures (time zero), and subsequently at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, and 130 minutes after seizure induction by PTZ.

Independent groups of animals were submitted to tests to determine the potential intrinsic effect of each pharmacological pretreatment of the NRM with atropine or mecamylamine followed by intraperitoneal treatment with physiological saline on nociceptive thresholds.

The effect of pretreatment of the NRM with atropine, mecamylamine, or physiological saline followed by intraperitoneal treatment with PTZ on interictal oscillation of the nociceptive thresholds was also investigated.

Finally, a sham procedure was performed in an independent group of rodents. This sham procedure consisted of the introduction of an injector needle inside the guide cannula inserted into the NRM without subsequent microinjections of cholinergic pharmacological antagonists or vehicle.

2.4. Behavioral test

Animals were placed in a circular arena with walls made of transparent acrylic, which measured 60 cm in diameter and 50 cm in height. This apparatus was located in an experimental compartment and was illuminated by a fluorescent lamp (350 lx at the arena floor level). Evaluation of the effects of drug administration (PTZ, atropine, mecamylamine, and physiological saline) was performed while rats were inside the arena.

The tonic–clonic convulsive reactions induced by PTZ were the motor parameters used to evaluate the effect of GABA-mediated Cl^- influx blockade. Latency to seizures was defined as the time from injection of PTZ to the first evidence of anterior paw myoclonia, which is considered as the first evidence of seizure onset. The frequency and severity of seizures (according to Racine's index [34] as modified later by de Freitas [20]) during the convulsive process caused by intraperitoneal administration of PTZ were also recorded, as indicated in Table 1.

Table 1
Severity scale of generalized tonic–clonic convulsive reactions induced by intraperitoneal administration of PTZ (64 mg/kg), according to Racine's index of severity [34], later modified by R.L. de Freitas [20].

Score	Seizing reaction
0.0	Exploratory behavior
1.0	Jaw and/or facial myoclonic action Short-duration anterior paw myoclonus
2.0	Head myoclonia Moderate myoclonia of anterior paws with duration of at least 5 s
3.0	Anterior limb tonic extension Anterior paw severe myoclonia with duration of at least 10 s
4.0	Complete tonic extension Rearing and severe myoclonia of anterior paw
5.0	Complete tonic extension Rearing and falling, myoclonia of anterior and posterior paws

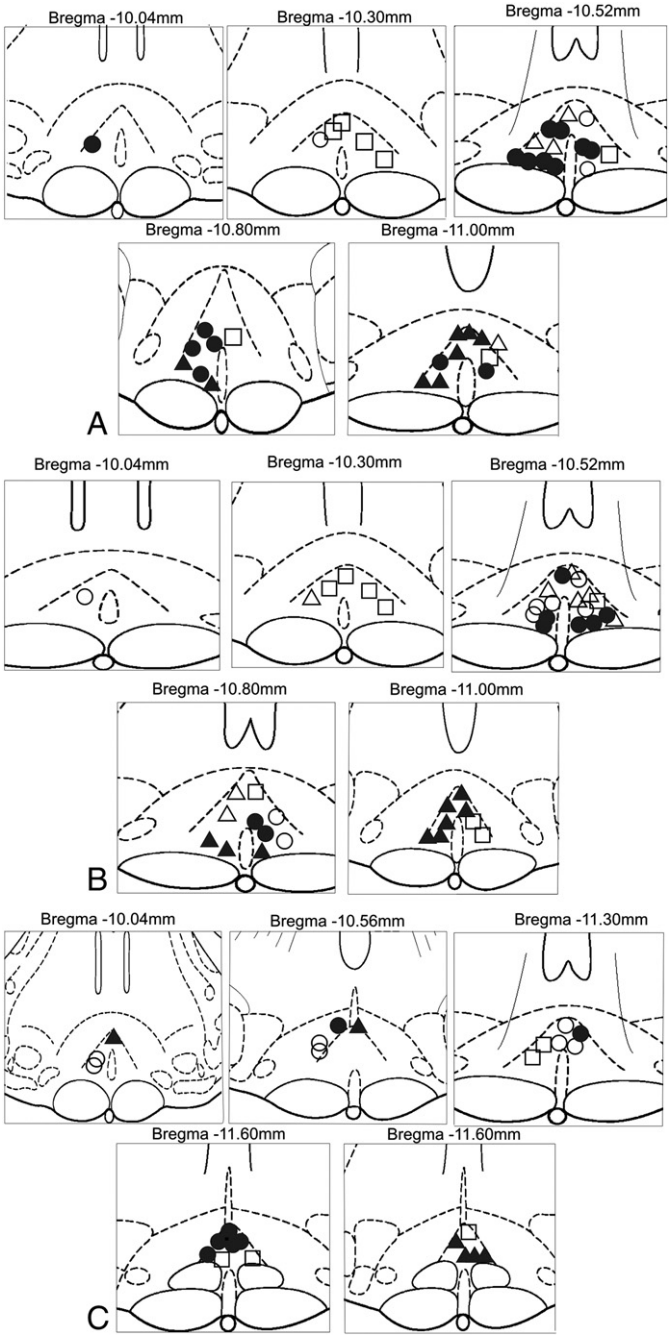

Fig. 1. A: Schematic representation of histologically confirmed sites of atropine microinjections at 1 μg/0.2μL (△), 3 μg/0.2 μL (●) and 5 μg/0.2 μL (○), sham procedure (□) and microinjections of physiological saline (▲) in the nucleus raphe magnus (NRM) followed by intraperitoneal (IP) treatment with pentylenetetrazole (PTZ) depicted in anagrams based on the atlas by Paxinos and Watson [36]. B: Schematic representation of histologically confirmed sites of microinjection of mecamylamine at 1 μg/0.2 μL (△), 3 μg/0.2 μL (●) and 5 μg/0.2 μL (○), sham procedure (□) and microinjections of physiological saline (▲) in the NRM followed by IP treatment with PTZ, depicted in anagrams based on the atlas by Paxinos and Watson [36]. C: Schematic representation of histologically confirmed sites of the sham procedure (□) and pretreatment of the NRM with microinjections of atropine at 5 μg/0.2 μL (●), mecamylamine at 5 μg/0.2 μL (○), and physiological saline (▲) followed by IP treatment with physiological saline, depicted in anagrams based on the atlas by Paxinos and Watson [36].

2.5. Drugs

Pentylenetetrazole (64 mg/kg), atropine, and mecamylamine were from Sigma/Aldrich, St. Louis, MO, USA. Each cholinergic antagonist was dissolved in physiological saline (NaCl 0.9%) shortly before administration and administered at the following concentrations: 1 μg/0.2 μL, 3 μg/0.2 μL, and 5 μg/0.2 μL. Physiological saline was used as the control.

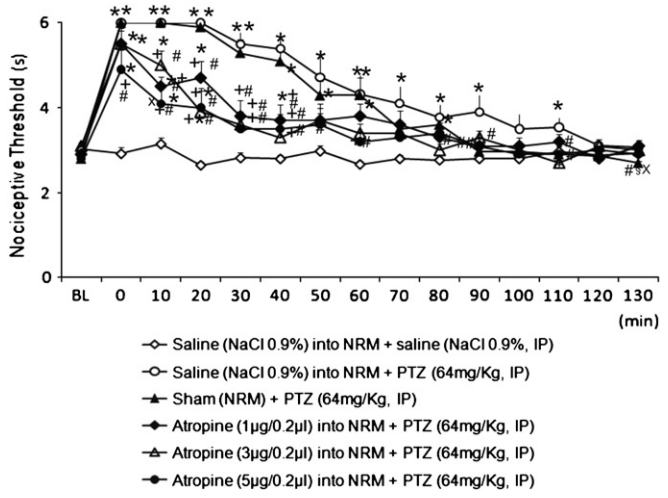

Fig. 2. Effect of microinjections of atropine (1 μg/0.2 μL, 3 μg/0.2 μL and 5 μg/0.2 μL) in the nucleus raphe magnus (NRM) (N = 8) on postictal antinociception. Data were presented as mean SEM. *, statistically significant differences ($p<0.05$), as compared with the saline (NaCl 0.9%)-NRM pretreatment followed by intraperitoneal (IP) treatment with physiological saline group; #, statistically significant differences ($p<0.05$) as compared with the NRM saline (NaCl 0.9%)-pretreatment followed by IP treatment with pentylenetetrazole (PTZ) (64 mg/kg) group; +, statistically significant differences ($p<0.05$) compared to the sham procedure in the NRM followed by IP treatment with PTZ (64 mg/kg) group; §, statistically significant differences ($p<0.05$) compared to the NRM atropine at 1 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group; X, statistically significant differences ($p<0.05$) as compared with NRM atropine at 3 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group; &, statistically significant differences ($p<0.05$) as compared with NRM atropine at 5 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group, according to repeated measure ANOVA, followed by Duncan´s post hoc test.

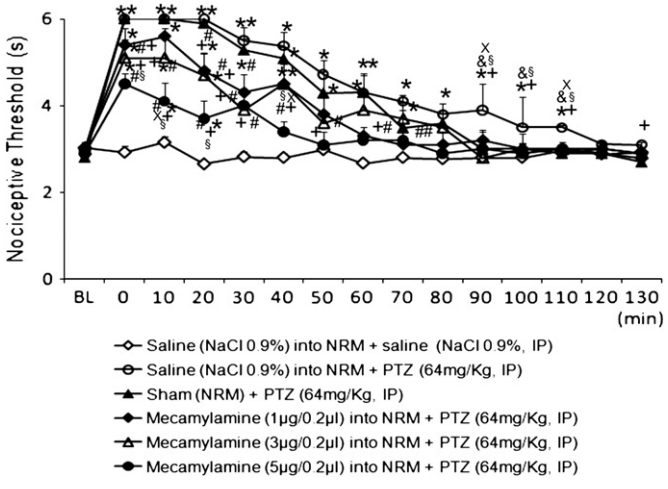

Fig. 3. Effect of microinjections of mecamylamine (1 μg/0.2 μL, 3 μg/0.2 μL and 5 μg/0.2 μL) in the NRM (N = 8) on postictal antinociception. Data were presented as mean SEM. *, statistically significant differences ($p<0.05$), as compared with the saline (NaCl 0.9%)-NRM pretreatment followed by intraperitoneal (IP) treatment with physiological saline group; #, statistically significant differences ($p<0.05$) as compared with the NRM saline (NaCl 0.9%)-pretreatment followed by IP treatment with pentylenetetrazole (PTZ) (64 mg/kg) group; +, statistically significant differences ($p<0.05$) as compared with the sham procedure in the NRM followed by IP treatment with PTZ (64 mg/kg) group; §, statistically significant differences ($p<0.05$) as compared to the NRM mecamylamine at 1 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group; X, statistically significant differences ($p<0.05$) as compared with the NRM mecamylamine at 3 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group; &, statistically significant differences ($p<0.05$) as compared with NRM mecamylamine at 5 μg/0.2 μL-pretreatment followed by IP treatment with PTZ (64 mg/kg) group, according to repeated-measurement ANOVA, followed by Duncan´s post-hoc test.

2.6. Histological analysis

After testing, the rats were anesthetized with sodium pentobarbital (45 mg/kg, ip) and perfused via the left ventricle. The blood was washed out with cold, oxygen-enriched, Ca^{2+}-free Tyrode's buffer (40 mL at 4 °C), followed by 200 mL of ice-cold 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, for 15 minutes at a pressure of 50 mm Hg. The brainstem was quickly sectioned, removed, and immersed in fresh fixative for 4 hours at 4 °C. It was then rinsed in 10 and 20% sucrose dissolved in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C, for at least 12 hours in each solution. Tissue pieces were immersed in 2-methylbutane (Sigma), frozen on dry ice, embedded in Tissue Tek OCT, and cut with a cryostat (CM 1950 Leica) at −22 °C. Sections were then mounted on glass slides coated with chrome alum gelatin to prevent detachment and stained with haematoxylin–eosin (LEICA Autostainer XL, CV 5030) to be viewed on a photomicroscope (AxioImager Z1, Zeiss). Statistical analysis was performed exclusively with data from the animals that presented signs that microinjection into the NRM had been successful.

2.7. Analysis of results

Data from experiments investigating the effect of cholinergic receptor blockade in the NRM on TFLs and interictal and postictal antinociception were submitted to repeated-measures analysis of variance (ANOVA). In the case of a significant treatment × time interaction, one-way ANOVA was performed at each time interval, followed by Duncan's post hoc test. Comparisons between tail-flick baseline values and TFLs recorded immediately after each pharmacological pretreatment of the NRM and those recorded before intraperitoneal treatment with PTZ were analyzed with Wilcoxon's test. $P<0.05$ was indicative of statistically significant differences.

3. Results

Pentylenetetrazole induced severe tonic–clonic seizures in all animals, followed by a sustained increase in the nociceptive threshold lasting 110 minutes after convulsive reactions, and these reactions

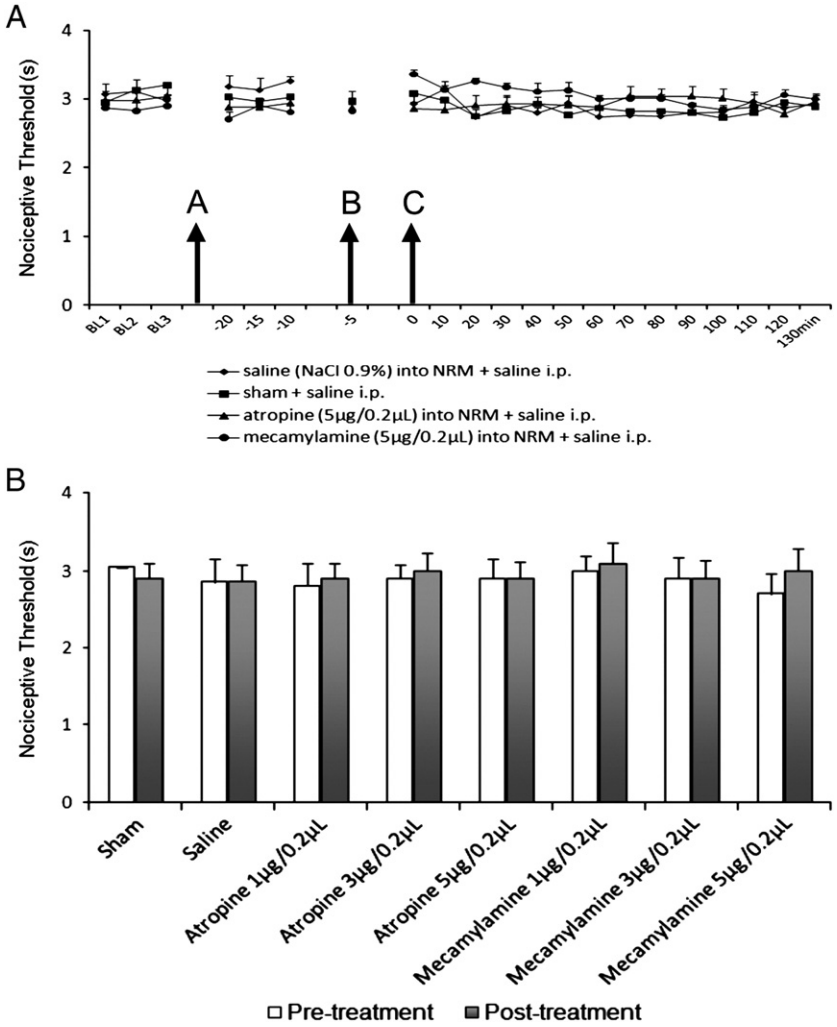

Fig. 4. A: Lack of effect of NRM pretreatment with physiological saline (N = 7), NRM sham procedure (N = 6), NRM atropine at 5 µg/0.2 µL-pretreated group (N = 7) or NRM mecamylamine at 5 µg/0.2 µL-pretreated group (N = 7), followed by IP administration of physiological saline on the nociceptive thresholds, which were recorded as withdrawal reflexes in the tail-flick test. Data were presented as mean ± SEM. P > 0.05 in all cases, according to repeated measure ANOVA, followed by Duncan´s post hoc test. Arrows in A represent the stereotaxic surgery, in B the administration of cholinergic antagonists and physiological saline in the ventromedial medulla, and in C the IP administration of physiological saline 5 min after each NRM pretreatment. B: Lack of effect of the sham procedure, atropine, mecamylamine or physiological pretreatment of the NRM on nociceptive thresholds, according to Student's Wilcoxon test. Columns represent means; bars represent SEM.

were not preceded by wild running. All sites studied were situated inside the NRM (Figs. 1A, B and C).

Microinjections of atropine (1 μg/0.2 μL, 3 μg/0.2 μL, and 5 μg/0.2 μL) into the NRM (Fig. 1A) decreased postictal antinociception. Significant effects of treatment [$F(5,40) = 15.27$, $P<0.001$], time [$F(14,27) = 40.07$, $P<0.001$], and the treatment × time interaction [$F(70,127) = 3.18$, $P<0.001$] were observed. Repeated-measures ANOVA revealed a significant effect of NRM pretreatment with the cholinergic muscarinic receptor antagonist atropine from 0 to 130 minutes [$F(5,40)$ varying from 1.21 to 17.77, $P<0.05$]. Post hoc analyses showed that microinjections of atropine into the NRM decreased the postictal analgesia recorded immediately after the seizure and 10 to 100 minutes after convulsive reactions (Duncan´s post hoc test, $P<0.05$ in all cases) (Fig. 2).

Microinjections of mecamylamine (1 μg/0.2 μL, 3 μg/0.2 μL, and 5 μg/0.2 μL) into the NRM (Fig. 1B) also decreased the phenomenon of tonic–clonic seizure-induced antinociception. Statistical analysis revealed significant effects of treatment [$F(5,40) = 12.71$, $P<0.001$], time [$F(14,27) = 34.09$, $P<0.001$], and the treatment × time interaction [$F(70,127) = 2.11$, $P<0.001$]. Repeated-measures ANOVA showed a significant effect of NRM pretreatment with the cholinergic nicotinic receptor antagonist mecamylamine on postictal analgesia from 0 to 130 minutes [$F(5,40)$ varying from 0.81 to 15.82, $P<0.05$]. Post hoc analyses showed that microinjections of mecamylamine into the NRM caused a decrease in the postictal analgesia recorded immediately after convulsive responses and from 10 to 70 minutes after seizures (Duncan's post hoc test, $P<0.05$), as shown in Fig. 3.

Interictal oscillations of nociceptive thresholds were observed to increase in height after convulsive reactions caused by PTZ. Pretreatment of the NRM with atropine or mecamylamine (5 μg/0.2 μL) also antagonized interictal antinociception, which was most pronounced in later interictal measurements. There were significant effects of treatment [$F(3,23) = 15.34$, $P<0.001$], time [$F(3,21) = 24.01$, $P<0.001$], and the treatment × time interaction [$F(9,59) = 6.33$, $P<0.001$]. Repeated-measures ANOVA revealed a significant effect of NRM pretreatment with cholinergic muscarinic and nicotinic receptor antagonists on interictal antinociception [$F(3,23)$ varying from 4.24 to 17.31, $P<0.05$]. Post hoc analyses showed that microinjections of atropine or mecamylamine into the NRM decreased interictal analgesia, especially during the later interictal period (Duncan´s post hoc test, $P<0.05$ in all cases) (Fig. 5).

Pharmacological treatment of the NRM with atropine or mecamylamine (5 μg/0.2 μL) had no statistically significant intrinsic effect on nociceptive thresholds, as shown in Fig. 4A. Repeated-measures ANOVA showed no significant effect of either treatment [$F(3,22) = 0.30$, $P>0.05$], time [$F(20,3) = 1.24$, $P>0.05$], or the treatment × time interaction [$F(60,5) = 2.1$, $P>0.05$]. Repeated-measures ANOVA did not reveal significant differences of NRM pretreatment with either atropine or mecamylamine 5 minutes before intraperitoneal administration of physiological saline, as determined by consecutive recordings from 0 to 130 minutes of withdrawal reflex latencies [$F(3,23)$ varying from 0.13 to 5.63, $P>0.05$].

In addition, the different pharmacological treatments of the NRM with atropine (Figs. 6A, B and C) and mecamylamine (Figs. 7A, B and C) had no statistically significant effects on the tail-flick baseline curve (Fig. 4B), the duration of or latency to pharmacologically induced seizures, or the severity or frequency of convulsive reactions, according to Racine's index of severity [34] as modified later by R.L. de Freitas [20], during the convulsive responses caused by intraperitoneal administration of PTZ (Duncan' s post hoc test, $P>0.05$ in all cases).

4. Discussion

The present findings suggest the participation of muscarinic receptors of NRM neurons in both interictal and postictal analgesia. It was found that atropine decreases postictal antinociception immediately after the end of seizures until 100 minutes after convulsions, in a dose-dependent manner at 10 minutes after seizure. Atropine also induced a decrease in the interictal antinociception in at least two different time windows following the pharmacological induction of seizures with PTZ.

This finding was corroborated by pretreatment of the NRM with mecamylamine, which resulted in an expressive antagonism of the postictal antinociception similar to that caused by

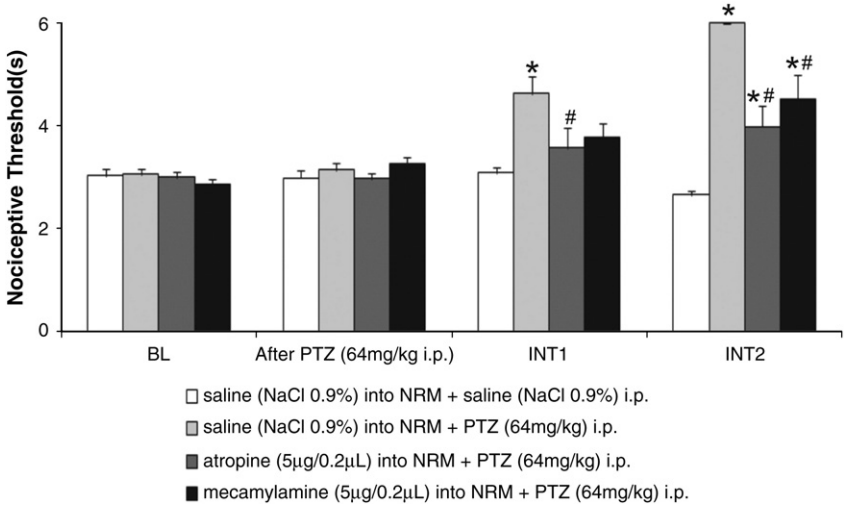

Fig. 5. Effect of NRM pretreatment with physiological saline (N = 7), atropine (5 μg/0.2 μL) (N = 7), or mecamylamine (5 μg/0.2 μL) (N = 7), on nociceptive baseline threshold and on the interictal antinociception recorded twice after IP treatment with PTZ, and lack of effect of the NRM pretreatment with physiological saline (N = 6) plus IP treatment with physiological saline on tail-flick latencies. Data are presented as mean SEM. *, statistically significant differences ($p<0.05$) as compared with NRM saline (NaCl 0.9%)-pretreated group + IP treatment with physiological saline; #, statistically significant differences ($p<0.05$) as compared with the NRM saline (NaCl 0.9%)-pretreated group + IP treatment with PTZ, according to repeated-measure ANOVA, followed by Duncan´s post-hoc test. Columns represent mean nociceptive threshold (NT); bars represent the SEM. INT: interictal nociceptive threshold. BL: average baseline values.

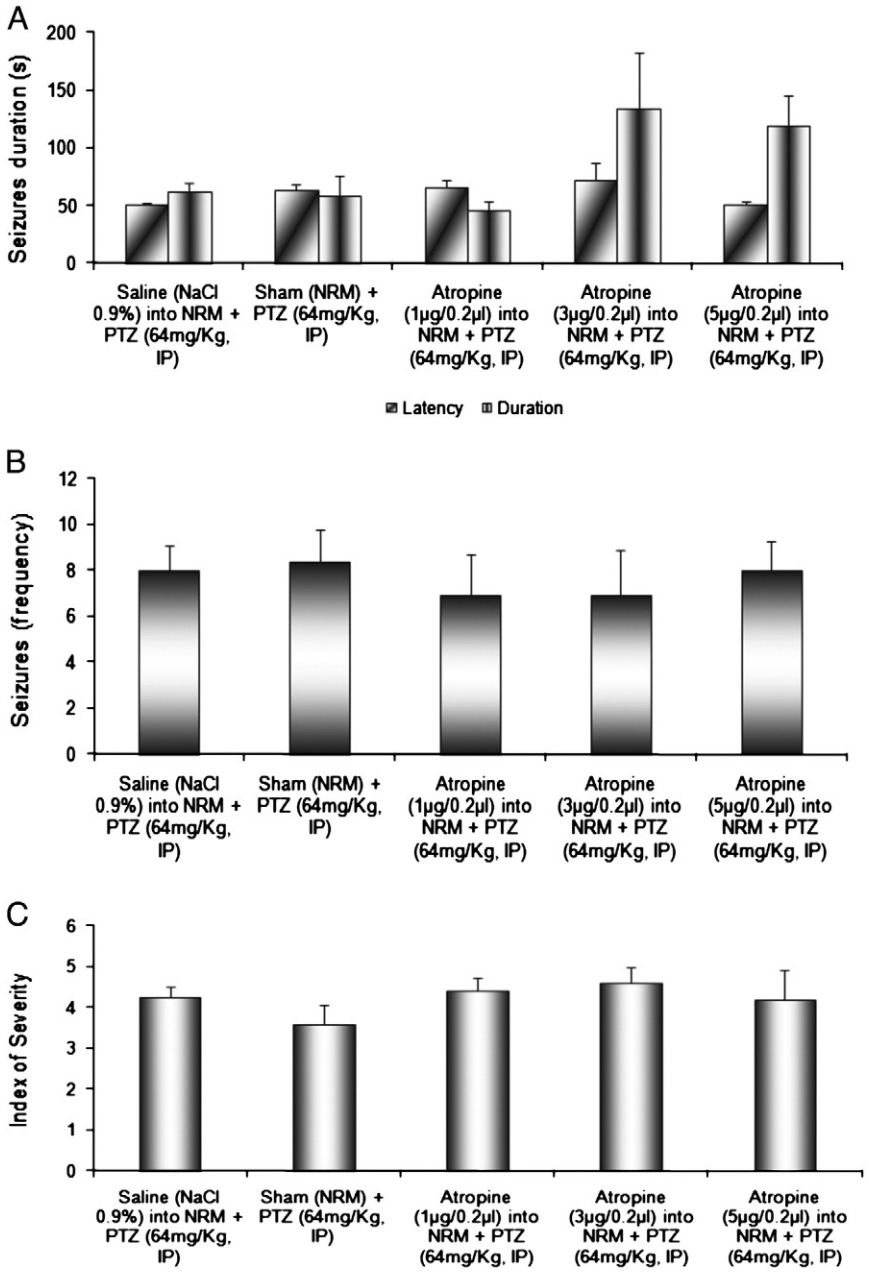

Fig. 6. A: Effect of nucleus raphe magnus (NRM) pretreatment with physiological saline + intraperitoneal (IP) treatment with pentylenetetrazole (PTZ) (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with atropine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the latency and duration of tonic–clonic seizures. B: Effect of NRM pretreatment with physiological saline + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with atropine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the frequency of tonic–clonic seizures. C: The effect of NRM pretreatment with physiological saline + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with atropine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with atropine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the severity of tonic–clonic seizures.

pretreatment of the NRM with atropine. Mecamylamine-pretreated animals exhibited a dose-dependent effect from 0 to 40 minutes after seizure onset. In addition, the blockade of cholinergic nicotinic receptors in the NRM also decreased the interictal antinociception recorded in a late time window period among seizures caused by the peripheral blockade of GABA-mediated Cl^- influx.

There was no intrinsic effect of pretreatment of the NRM with cholinergic antagonists, considering that pretreatment of the NRM with atropine or mecamylamine failed to alter baseline TFLs or the severity, duration, latency, or frequency of convulsive seizures, in comparison to controls. In addition, central administration of atropine or mecamylamine into the NRM at the highest concentration (5 μg/0.2 μL), followed by intraperitoneal treatment with physiological saline, did not have a statistically significant effect on baseline nociceptive thresholds.

Previous studies have reported that a lesion in the NRM significantly decreases the increase in nociceptive threshold caused by tonic–clonic seizures [3]. A similar effect was observed with respect to the analgesia induced by morphine after lesions to the NRM [18,35]. However, we cannot rule out the involvement of other cholinergic neuron- and cholinergic fiber-containing structures of the

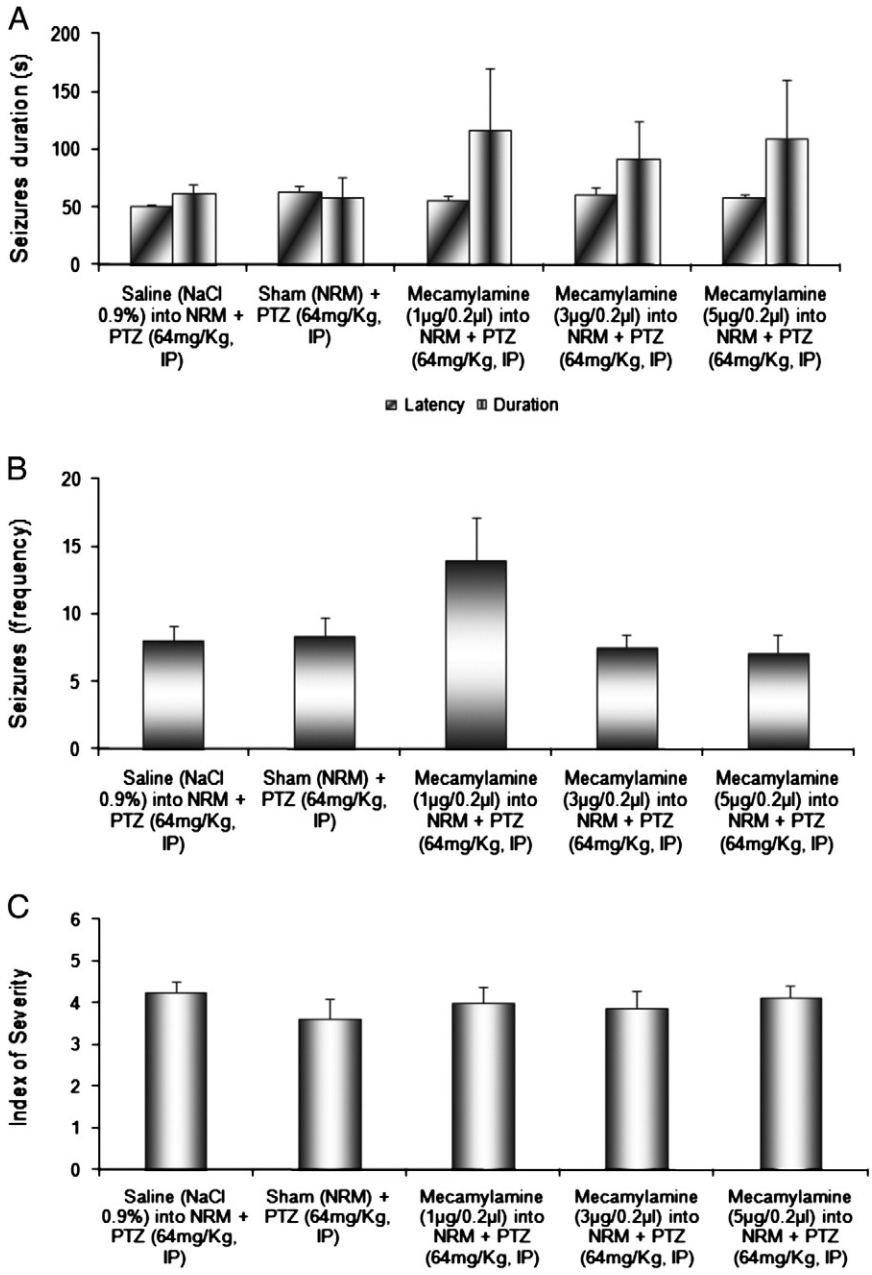

Fig. 7. A: Effect of nucleus raphe magnus (NRM) pretreatment with physiological saline + intraperitoneal (IP) treatment with pentylenetetrazole (PTZ) (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with mecamylamine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the severity of tonic–clonic seizures. B: Effect of NRM pretreatment with physiological saline + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with mecamylamine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the frequency of tonic–clonic seizures. C: Effect of NRM pretreatment with physiological saline + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 1 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8); NRM-pretreatment with mecamylamine at 3 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) and NRM-pretreatment with mecamylamine at 5 μg/0.2 μL + IP PTZ (64 mg/kg) (N = 8) on the latency and duration of tonic–clonic seizures.

brainstem, such as the pedunculopontine tegmental nucleus, the periaqueductal gray matter, or serotonergic neurons of the dorsal raphe nucleus in the organization of postictal analgesia.

The role of serotoninergic receptors in this antinociceptive phenomenon is already known [3]; however, this is the first time in which participation of muscarinic and nicotinic receptors of the NRM in elaboration of interictal and postictal antinociception has been demonstrated. It is possible that involvement of the cholinergic system in interictal and postictal antinociception is based on the control of activity of serotonergic neurons of the NRM that send output to the secondary ascending neurons of the spinal cord and trigeminal spinalis nucleus.

Previous studies have used peripheral administration of cholinergic antagonists to demonstrate involvement of the cholinergic systems in the analgesia induced by convulsive reactions [20], suggesting that acetylcholine is released at the synaptic connection of neural networks responsible for the elaboration of postictal analgesia, in the early stages of the antinociceptive phenomenon. However, serotonin and noradrenaline are critically released in later periods of postictal analgesia [21]. Ascending cholinergic pathways

from the pedunculopontine tegmental nucleus, with synaptic targets in the dorsal raphe nucleus, and cholinergic pathways descending toward the NRM and locus coeruleus appear to play an important role in the postictal antinociceptive phenomenon [29].

In conclusion, the data described here show that cholinergic nicotinic and muscarinic receptor antagonists administered into the NRM cause long-lasting blockade of the antinociception induced by tonic–clonic seizures, demonstrating the relevance of cholinergic inputs to the NRM in the modulation of postictal antinociception. Therefore, the present work takes an additional step in this important line of research, demonstrating the involvement of NRM neurons in the organization of both interictal and postictal antinociception.

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